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## ORIGINAL PAPER

# *Aureobasidium melanogenum*: a native of dark biofinishes on oil treated wood

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**Abstract** The genus *Aureobasidium*, which is known as a wood staining mould, has been detected on oil treated woods in the specific stain formation called biofinish. This biofinish is used to develop a new protective, self-healing and decorative biotreatment for wood. In order to understand and control biofinish formation on oil treated wood, the occurrence of different *Aureobasidium* species on various wood surfaces was studied. Phenotypic variability within *Aureobasidium* strains presented limitations of morphological identification of *Aureobasidium* species. PCR amplification and Sanger sequencing of ITS and *RPB2* were used to identify the culturable *Aureobasidium* species composition in mould stained wood surfaces with and without a biofinish. The analysed isolates showed that several *Aureobasidium* species were present and that *Aureobasidium melanogenum* was predominantly detected, regardless of the

presence of a biofinish and the type of substrate. *A. melanogenum* was detected on wood samples exposed in the Netherlands, Cameroon, South Africa, Australia and Norway. ITS-specific PCR amplification, cloning and sequencing of DNA extracted from biofinish samples confirmed results of the culturing based method: *A. melanogenum* is predominant within the *Aureobasidium* population of biofinishes on pine sapwood treated with raw linseed oil and the outdoor placement in the Netherlands.

**Keywords** *Aureobasidium pullulans* · Linseed oil · Mould staining · Pine · Sustainable · Wood protection

## Introduction

*Aureobasidium* are wood staining fungi, in particular on wood situated outdoors above the ground (Dickinson 1972; Dix and Webster 1995; Bardage 1998; Schmidt 2006; Gobakken and Westin 2008). The interest in *Aureobasidium* has recently increased, because of its role in the formation of biofinishes on wood (Sailer et al. 2010; van Nieuwenhuijzen et al. 2013; van Nieuwenhuijzen et al. 2015; Filippovych et al. 2015). The term biofinish was introduced for a uniform dark mould covering which emerged outdoors on oil treated wood (van Nieuwenhuijzen et al. 2015). Although the protection mechanism and durability of this biofinish is still under investigation, biofinished

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wood is considered to be an appealing biocide-free construction material that has the advantage of also having self-healing properties.

Until now it is unknown which *Aureobasidium* species participates in the biofinish formation and whether a biofinish is composed of more than a single species. Although *Aureobasidium* has been isolated from many organic and inorganic substrates and geographical locations (Zalar et al. 2008; Slepecky and Starmer 2009; Gaur et al. 2010), the ubiquity of the specific species is unknown. The geographical location, the combination of wood species and the oil treatment may all have an impact on the species composition of the *Aureobasidium* population in biofinishes. Species-specific behaviour, such as phenotype and physiology (Samson et al. 2010; Houbraeken 2013), should be included in future research in order to understand and control dark mould growth on oil treated wood. Therefore insight in the species composition of the biofinish is highly relevant.

The ascomycete genus *Aureobasidium* is a member of the family *Aureobasidiaceae* within the class of the *Dothideomycetes* (Thambugala et al. 2014; Wijayawardene et al. 2014). *Kabatiella* is closely related to *Aureobasidium* based on morphology and phylogeny (Zalar et al. 2008; Bills et al. 2012; Crous et al. 2011; Peterson et al. 2013; Thambugala et al. 2014) and some of these *Kabatiella* species may belong to *Aureobasidium* (Peterson et al. 2013; Thambugala et al. 2014). In addition future studies may result in the transfer of the species *Selenophoma mahoniae* and *Columnosphaeria (Discosphaerina) fagi* into *Aureobasidium* (Yurlova et al. 1999; Peterson et al. 2013; Thambugala et al. 2014). A well-known *Aureobasidium* species is *Aureobasidium pullulans* (Zalar et al. 2008; Gostinčar et al. 2014). The total number of classified *Aureobasidium* species currently varies per database, for example 38 in MycoBank and 13 in GenBank (October 2015).

Before DNA sequencing was applied in fungal taxonomy, the species classification system was mainly based on physiologic and phenotypic characteristics. In the case of *Aureobasidium*, colony pigmentation was used as a species-specific phenotypic characteristic (Zalar et al. 2008; Peterson et al. 2013). Nowadays, also the genealogical concordance phylogenetic species recognition (GCPSR) concept is commonly applied for species delimitation (Taylor et al. 2000). For species delimitation according to

GCPSR multigene phylogenies are required. Next to the large subunit and the internal transcribed spacer regions (incl. 5.8S rDNA) (ITS) more variable genes such as translation elongation factor 1 $\alpha$ ,  $\beta$ -tubulin and RNA polymerase II- second largest subunit (*RPB2*) have been applied or recommended for phylogenetic analysis of *Aureobasidium* species (Zalar et al. 2008; Manitchotpisit et al. 2009; Peterson et al. 2013; Gostinčar et al. 2014). A phylogeny, including all described genera and species within the *Aureobasidiaceae*, is not yet available.

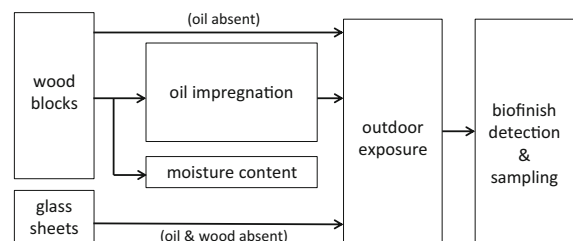
The ITS locus is assigned as the primary barcode for fungal species (Schoch et al. 2012). A large number of ITS barcode sequences of *Aureobasidium* species is available in public databases, which makes this DNA region a suitable marker to identify *Aureobasidium* (Manitchotpisit et al. 2009). To date, no second fungal barcode has been determined for a reliable *Aureobasidium* identification on species level.

The aim of this study was to explore the *Aureobasidium* species composition of biofinishes on wood. Culturable *Aureobasidium* isolates, retrieved from substrates with and without biofinishes, were identified. The wood species, oil treatments and exposure sites were related to the culturable species composition. Also direct extraction of biofinish DNA, followed by ITS amplification, cloning and sequencing were used to determine the species compositions of biofinishes.

## Materials and methods

### Substrates and outdoor exposure

Nine sample sets were analysed in this study. Each set contained oil treated wood samples. Untreated pine



**Fig. 1** Flowchart illustrating the experimental setup of this study

sapwood and glass were also selected for several sample sets (Fig. 1; Table 1), representing oil-free organic and inorganic materials that are associated with *Aureobasidium* growth (Gorbushina and Palinska 1999; Schabereiter-Gurtner et al. 2001; van Nieuwenhuijzen et al. 2015). The amount of different substrates (e.g. wood species, oil type), the geographical location of the outdoor exposure and exposure time was specific for each sample set (Table 1).

The wood species tested were pine (*Pinus sylvestris*) sapwood, spruce (*Picea abies*) and ilomba (*Pycnanthus angolensis*). No specific sapwood or heartwood selection was made for the latter two species. The surfaces of the wood samples were planed. The samples measured 5 cm (longitudinal axis),  $\times$  2.5 cm  $\times$  1.5 cm, except the specimens of set 4 which measured 10 cm  $\times$

14 cm  $\times$  2 cm. Glass sheets (Fisher Scientific) measured 10 cm  $\times$  10 cm  $\times$  0.3 cm.

Three different vegetable oil types were used to impregnate the wood specimens: raw linseed oil (Vereenigde Oliefabrieken; iodine value 183 and 0.81 % free fatty acids), olive oil (two brands: in case of sample set 4 unfiltered olive oil of 100 % Carolea olive, Calabrië EV Bio 2013; for the other sample sets Carbonel, extra vierge, iodine value 82 and 0.34 % free fatty acids), and stand linseed oil (Vliegenghart, viscosity P45). To determine the average moisture content right before impregnation, additional untreated test pieces of wood were dried at 105 °C. The moisture content of these wood pieces was up to 12 %. The impregnation of the small specimens (5 cm  $\times$  2.5 cm  $\times$  1.5 cm) was carried out using a

**Table 1** Overview of the sample sets used for outdoor exposure and their characteristics

| Samples | Substrate                      | Number of specimens | Locality                   | Exposure time |
|---------|--------------------------------|---------------------|----------------------------|---------------|
| Set 1   | Pine sapw. & raw linseed oil   | 2                   | Utrecht, The Netherlands   | 2 weeks       |
|         | Glass                          | 2                   |                            |               |
| Set 2   | Pine sapw. & raw linseed oil   | 2                   | Utrecht, The Netherlands   | 5 weeks       |
|         | Glass                          | 2                   |                            |               |
| Set 3   | Pine sapw. & raw linseed oil   | 2                   | Utrecht, The Netherlands   | 5 months      |
|         | Glass                          | 2                   |                            |               |
| Set 4   | Spruce & raw linseed oil       | 1                   | Utrecht, The Netherlands   | 12 months     |
|         | Ilomba & raw linseed oil       | 1                   |                            |               |
|         | Pine sapw. & raw linseed oil   | 1                   |                            |               |
|         | Pine sapw. & olive oil         | 1                   |                            |               |
|         | Pine sapw. & stand oil         | 1                   |                            |               |
|         | Untreated pine sapw            | 1                   |                            |               |
| Set 5   | Pine sapw. & raw linseed oil   | 1                   | Utrecht, The Netherlands   | 1.5 years     |
|         | Pine sapw. & olive oil         | 1                   |                            |               |
|         | Pine sapw. & stand linseed oil | 1                   |                            |               |
|         | Ilomba & olive oil             | 1                   |                            |               |
|         | Spruce & olive oil             | 1                   |                            |               |
| Set 6   | Spruce & raw linseed oil       | 1                   | Douala, Cameroon           | 8 months      |
|         | Ilomba & raw linseed oil       | 1                   |                            |               |
|         | Pine sapw. & raw linseed oil   | 1                   |                            |               |
|         | Pine sapw. & olive oil         | 1                   |                            |               |
|         | Untreated pine sapw            | 1                   |                            |               |
| Set 7   | Same materials as set 6        |                     | Johannesburg, South Africa | 1.7 years     |
| Set 8   | Same materials as set 6        |                     | Adelaide, Australia        | 1.5 years     |
| Set 9   | Same materials as set 6        |                     | Ås, Norway                 | 2 years       |

vacuum time of 30 min at  $-1$  bar followed by 1 h pressure of 8 bar. A vacuum time of 1 h at  $-1$  bar followed by 2 h pressure of 8 bar was used for the larger specimens of sample set 4.

The wood samples of set 1–3 were steamed twice with hot air for 20 min on two consecutive days (European Standard 1996; Fritsche and Laplace 1999). The glass sheets were cleaned with alcohol and autoclaved before outdoor exposure. No sterilisation method was applied to the wood samples of set 4–9.

Five sites, located in different countries (Table 1) were used for outdoor exposure. The samples from set 4 remained outdoors during the biomass removal. Specifications on outdoor exposure and handling procedures were described in van Nieuwenhuijzen et al. (2015).

### Biofinish assessment

The samples of set 5 and 7–9 have previously been evaluated for biofinish formation in the study of van Nieuwenhuijzen et al. (2015). This method consisted of visual analysis of the stain coverage on the surface as well as in situ spectrophotometer measurements of the pigmentation. In short a biofinish was assigned when the stain coverage was above 90 % and the pigmentation, expressed by triplets as used in the sRGB colour space, met the following criteria: all the red (R), green (G) and blue (B) values were below 82 and the difference between two values of a single RGB triplet was below 20. In comparison the RGB triplet of ultimate black was [0,0,0] and ultimate white was [255,255,255]. The biofinish assessment was also applied on the samples of set 6. The presence of a biofinish on the wood samples of set 1–4 was determined according to the surface coverage part of the biofinish assessment. A full biofinish assessment of the samples of set 4 was performed three months after fungal isolation.

### Collection of *Aureobasidium* isolates

Within a sample set, up to two specimens per substrate were used for isolation (Table 1). The swab sampling method as described in van Nieuwenhuijzen et al. (2015) was used to collect biomass. Biomass suspensions were plated on malt extract agar (MEA) supplemented with penicillin and streptomycin (P/S)

and on dichloran 18 % glycerol agar (DG18). The formulation of the agar media were according to Samson et al. (2004) and the plates were incubated at 25 °C for 14 days. A selection of the colonies, that phenotypically resembled *Aureobasidium*, was transferred to new MEA plates. The phenotypic characteristics used to determine *Aureobasidium* colonies: fast growing, yeast like colonies with an irregular edge, either white/pale pink coloured colonies mostly with a black centre and/or sectors or black coloured colonies with a small white boundary; white aerial hyphae sometimes present. Isolates of the selected colonies were deposited in the working collection of the Department of Applied and Industrial Mycology (DTO) housed at the CBS-KNAW Fungal Biodiversity Centre.

### Phenotypic diversity of *Aureobasidium* strain DTO 217-G5

A large phenotypic variation within the *Aureobasidium* colonies on agar plates was observed during isolation. The isolate DTO 217-G5 (= CBS 140241) was used to study the phenotypic variability of a single strain. It was selected as a representative of the black cultures obtained from oil treated wood in the initial stage of biofinish formation. At first biomass was obtained of the edge of a 7 days old colony on MEA and washed in ultrapure water twice before dilution in ultrapure water. This dilution was combined with  $10 \times$  Yeast Nitrogen Base (Difco Laboratories 1998) with no additional carbon source and transferred to a shake flask. Due to the limited amount of carbon, the strain was cultivated in a nutrient limited and therefore stressful environment. After 24 h of shaking at 175 rpm at 25 °C, a serial dilution was made of the cell suspension and plated on oatmeal agar. After 5 days of incubation at 25 °C, four phenotypically diverse colony forming units (CFU's) were selected as parental colonies and inoculated on MEA P/S (first MEA P/S inoculation). After incubation each colony was transferred to a new MEA P/S plate in triplicate. These colonies on the new plates were again transferred to MEA P/S three times in succession. Phenotypically diverse areas were selected for the biomass transfers. Photos were made and ITS sequences generated (as described below) of the colonies of the first and last inoculation on MEA P/S.

## DNA extraction, amplification and sequencing

Isolates were grown on MEA plates prior to DNA extraction. DNA was extracted using the Ultraclean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The ITS and *RPB2* fragments were amplified using the primer pairs V9G (de Hoog and Gerrits van den Ende 1998) & LS266 (Masclaux et al. 1995) and RPB-PenR1 & RPB-PenR2 (Manitchotpisit et al. 2009). The PCR reactions were performed according to van Nieuwenhuijzen et al. (2015). The *RPB2*-PCR program differed by a primer annealing at 54 °C for 60 s. The amplified DNA fragments were sequenced and assembled as described in Yilmaz et al. (2014). Generated sequences are deposited in GenBank. The accession numbers of the new outdoor isolates are included in Table 2.

## Phylogenetic analysis and identification of isolates

Reference strains of species which were used to generate a benchmark for the molecular identification of the *Aureobasidium* isolates are listed in Table 3. The GenBank accession numbers of the sequences are included in the table, except for the sequences of the *Aureobasidium thailandense* strains generated by Peterson et al. (2013; TreeBASE SN4236). The ITS and *RPB2* sequence data sets were aligned using the program Muscle within MEGA version 5 (Tamura et al. 2011). Maximum Likelihood (ML) analysis was performed using MEGA. The number of bootstrap replicates was set on 1000. *Sydowia polyspora* CBS 750.71 was selected as outgroup. The isolates were identified based on the clustering in the phylogenetic trees with the type and other representative strains. A bootstrap value of 70 % or more was considered as moderated support for the identification of clades.

## PCR, cloning and sequencing of biofinish DNA

ITS-specific cloning libraries were made of biofinishes of two types of substrates of set 5 in triplicate: pine sapwood & raw linseed oil (library PRL.1–PRL.3) and pine sapwood & olive oil (library PO.1–PO.3). An area of 2.5 cm × 2.5 cm of the upper surface of a specimen was scratched with a scalpel and DNA was extracted of the obtained biomass. The DNA extraction method, ITS primers and PCR-program were identical to the method

described above. The PCR master mixes with ITS primers were prepared with the GoTaq Long PCR Master Mix (Progenia) according to the manufacturer's instructions. The PCR products were purified with the QIAquick PCR purification kit. Following the manufacturer's instructions, 45 ng of PCR products was ligated and cloned (pGEM<sup>®</sup>-T Easy Vector Systems) into an *Escherichia coli* plasmid library. After growing ITS containing competent cells on plate, colonies were aseptically transferred to 10 µl demineralised water. PCR reactions were performed in 25 µl reaction mixtures containing 3 µl aliquots with ITS DNA, 2.5 µl PCR buffer, 2 µl MgCl<sub>2</sub> (25 mM), 11 µl demineralised sterile water, 5 µl dNTP (1 mM), 0.50 µl of each primer (10 µM) and 0.5 µl Taq polymerase (5 U/µl, Biotline). The ITS-PCR program, sequencing, and assembling were similar to the previously described method. Assembled ITS sequences were generated of 62–69 cloned colonies per wood sample library. The ITS sequences of the cloning libraries were screened against the non-redundant NCBI database, using the program BLASTN. Sequences resulting in hits with an identity of 97 % or more compared to *Aureobasidium* sequences of the database were used for phylogenetic analysis. Sequences were submitted to GenBank (Table 4).

## Results

### Biofinish assessment

All wood samples of sample set 3–9 showed dark discolorations, but a biofinish was only established on a few samples (Table 5). Biofinishes were detected on specific samples exposed for more than one year at the sample site in the Netherlands (sample set 4 and 5): pine sapwood samples treated with raw linseed oil or olive oil and spruce and ilomba samples treated with olive oil. Furthermore, biofinishes were detected on the pine sapwood sample treated with olive oil that was exposed in South Africa (sample set 7) and the pine sapwood sample treated with raw linseed oil that was exposed in Norway (sample set 9).

### Collection of *Aureobasidium* isolates

The number of isolates used in this study varied per substrate of each set (Table 5). These isolates were

**Table 2** Outdoor isolates obtained in this study and the GenBank accession numbers of their ITS and *RPB2* sequences

| Isolate    |         | GenBank accession no. |             | Isolate    |         | GenBank accession no. |             |
|------------|---------|-----------------------|-------------|------------|---------|-----------------------|-------------|
| DTO no.    | CBS no. | ITS                   | <i>RPB2</i> | DTO no.    | CBS no. | ITS                   | <i>RPB2</i> |
| DTO 212-D8 | –       | KT693505              | KT693748    | DTO 317-A6 | –       | KT693616              | KT693859    |
| DTO 212-F1 | –       | KT693506              | KT693749    | DTO 317-A7 | –       | KT693617              | KT693860    |
| DTO 212-G1 | –       | KT693507              | KT693750    | DTO 317-A8 | –       | KT693618              | KT693861    |
| DTO 212-I7 | –       | KT693508              | KT693751    | DTO 317-A9 | –       | KT693619              | KT693862    |
| DTO 213-A2 | –       | KT693509              | KT693752    | DTO 317-B1 | –       | KT693620              | KT693863    |
| DTO 213-A9 | –       | KT693510              | KT693753    | DTO 317-B2 | –       | KT693621              | KT693864    |
| DTO 214-C3 | –       | KT693511              | KT693754    | DTO 317-B3 | –       | KT693622              | KT693865    |
| DTO 214-C4 | –       | KT693512              | KT693755    | DTO 317-B4 | –       | KT693623              | KT693866    |
| DTO 214-C8 | –       | KT693513              | KT693756    | DTO 317-B5 | –       | KT693624              | KT693867    |
| DTO 214-D1 | –       | KT693514              | KT693757    | DTO 317-B6 | –       | KT693625              | KT693868    |
| DTO 214-D8 | –       | KT693515              | KT693758    | DTO 317-B8 | –       | KT693626              | KT693869    |
| DTO 214-D9 | –       | KT693516              | KT693759    | DTO 317-B9 | –       | KT693627              | KT693870    |
| DTO 228-C6 | –       | KT693517              | KT693760    | DTO 317-C1 | –       | KT693628              | KT693871    |
| DTO 212-H1 | –       | KT693518              | KT693761    | DTO 317-C2 | –       | KT693629              | KT693872    |
| DTO 212-H2 | –       | KT693519              | KT693762    | DTO 277-B5 | –       | KT693630              | KT693873    |
| DTO 214-E2 | –       | KT693520              | KT693763    | DTO 277-B6 | –       | KT693631              | KT693874    |
| DTO 214-E3 | –       | KT693521              | KT693764    | DTO 277-B7 | –       | KT693632              | KT693875    |
| DTO 214-F3 | –       | KT693522              | KT693765    | DTO 277-B8 | –       | KT693633              | KT693876    |
| DTO 214-F4 | –       | KT693523              | KT693766    | DTO 277-B9 | –       | KT693634              | KT693877    |
| DTO 214-G4 | –       | KT693524              | KT693767    | DTO 277-C1 | –       | KT693635              | KT693878    |
| DTO 214-G5 | –       | KT693525              | KT693768    | DTO 277-C2 | –       | KT693636              | KT693879    |
| DTO 214-I2 | –       | KT693526              | KT693769    | DTO 277-C3 | –       | KT693637              | KT693880    |
| DTO 214-I4 | –       | KT693527              | KT693770    | DTO 277-C4 | –       | KT693638              | KT693881    |
| DTO 214-I5 | –       | KT693528              | KT693771    | DTO 277-C5 | –       | KT693639              | KT693882    |
| DTO 215-B3 | –       | KT693529              | KT693772    | DTO 277-C6 | –       | KT693640              | KT693883    |
| DTO 215-B9 | –       | KT693530              | KT693773    | DTO 277-C7 | –       | KT693641              | KT693884    |
| DTO 215-C2 | –       | KT693531              | KT693774    | DTO 277-G5 | –       | KT693642              | KT693885    |
| DTO 215-C3 | –       | KT693532              | KT693775    | DTO 277-G6 | –       | KT693643              | KT693886    |
| DTO 215-D8 | –       | KT693533              | KT693776    | DTO 277-F4 | 140247  | KT693644              | KT693887    |
| DTO 215-E3 | –       | KT693534              | KT693777    | DTO 277-F5 | 140248  | KT693645              | KT693888    |
| DTO 217-F3 | –       | KT693535              | KT693778    | DTO 277-F6 | –       | KT693646              | KT693889    |
| DTO 217-F4 | –       | KT693536              | KT693779    | DTO 277-F7 | –       | KT693647              | KT693890    |
| DTO 217-F5 | –       | KT693537              | KT693780    | DTO 277-F8 | –       | KT693648              | KT693891    |
| DTO 217-G4 | 140240  | KT693538              | KT693781    | DTO 277-F9 | –       | KT693649              | KT693892    |
| DTO 217-G5 | 140241  | KT693539              | KT693782    | DTO 277-G1 | –       | KT693650              | KT693893    |
| DTO 217-H2 | –       | KT693540              | KT693783    | DTO 277-G2 | –       | KT693651              | KT693894    |
| DTO 218-A8 | –       | KT693541              | KT693784    | DTO 277-G3 | –       | KT693652              | KT693895    |
| DTO 218-B9 | –       | KT693542              | KT693785    | DTO 277-G4 | –       | KT693653              | KT693896    |
| DTO 218-D1 | –       | KT693543              | KT693786    | DTO 227-C6 | –       | KT693654              | KT693897    |
| DTO 218-F5 | –       | KT693544              | KT693787    | DTO 227-C7 | –       | KT693655              | KT693898    |
| DTO 218-F7 | –       | KT693545              | KT693788    | DTO 227-C8 | –       | KT693656              | KT693899    |
| DTO 218-G1 | –       | KT693546              | KT693789    | DTO 227-D3 | –       | KT693657              | KT693900    |
| DTO 218-G4 | –       | KT693547              | KT693790    | DTO 227-D4 | –       | KT693658              | KT693901    |

**Table 2** continued

| Isolate    |         | GenBank accession no. |             | Isolate    |         | GenBank accession no. |             |
|------------|---------|-----------------------|-------------|------------|---------|-----------------------|-------------|
| DTO no.    | CBS no. | ITS                   | <i>RPB2</i> | DTO no.    | CBS no. | ITS                   | <i>RPB2</i> |
| DTO 218-G6 | –       | KT693548              | KT693791    | DTO 227-D5 | –       | KT693659              | KT693902    |
| DTO 218-G8 | –       | KT693549              | KT693792    | DTO 227-D7 | 140249  | KT693660              | KT693903    |
| DTO 218-H6 | –       | KT693550              | KT693793    | DTO 227-D8 | –       | KT693661              | KT693904    |
| DTO 218-H8 | –       | KT693551              | KT693794    | DTO 227-E3 | –       | KT693662              | KT693905    |
| DTO 218-I1 | –       | KT693552              | KT693795    | DTO 227-E4 | –       | KT693663              | KT693906    |
| DTO 228-D1 | –       | KT693553              | KT693796    | DTO 227-E6 | –       | KT693664              | KT693907    |
| DTO 218-I3 | 140242  | KT693554              | KT693797    | DTO 227-E7 | –       | KT693665              | KT693908    |
| DTO 218-I4 | 140243  | KT693555              | KT693798    | DTO 227-E8 | –       | KT693666              | KT693909    |
| DTO 219-A4 | –       | KT693556              | KT693799    | DTO 285-D3 | –       | KT693667              | KT693910    |
| DTO 219-B9 | –       | KT693557              | KT693800    | DTO 285-D4 | –       | KT693668              | KT693911    |
| DTO 219-D5 | –       | KT693558              | KT693801    | DTO 285-D5 | –       | KT693669              | KT693912    |
| DTO 219-E8 | –       | KT693559              | KT693802    | DTO 296-E8 | 140250  | KT693670              | KT693913    |
| DTO 219-E9 | –       | KT693560              | KT693803    | DTO 285-D6 | –       | KT693671              | KT693914    |
| DTO 219-G2 | –       | KT693561              | KT693804    | DTO 285-D7 | 140251  | KT693672              | KT693915    |
| DTO 219-H1 | –       | KT693562              | KT693805    | DTO 285-D8 | –       | KT693673              | KT693916    |
| DTO 219-I3 | –       | KT693563              | KT693806    | DTO 285-D9 | –       | KT693674              | KT693917    |
| DTO 219-B8 | –       | KT693564              | KT693807    | DTO 285-E1 | 140252  | KT693675              | KT693918    |
| DTO 219-F9 | –       | KT693565              | KT693808    | DTO 285-E2 | 140253  | KT693676              | KT693919    |
| DTO 219-B2 | –       | KT693566              | KT693809    | DTO 285-E3 | –       | KT693677              | KT693920    |
| DTO 232-D6 | –       | KT693567              | KT693810    | DTO 285-E4 | 140254  | KT693678              | KT693921    |
| DTO 232-D7 | –       | KT693568              | KT693811    | DTO 296-F6 | –       | KT693679              | KT693922    |
| DTO 232-E6 | –       | KT693569              | KT693812    | DTO 296-G3 | –       | KT693680              | KT693923    |
| DTO 232-E7 | –       | KT693570              | KT693813    | DTO 296-G4 | –       | KT693681              | KT693924    |
| DTO 232-H8 | –       | KT693571              | KT693814    | DTO 296-G5 | 140255  | KT693682              | KT693925    |
| DTO 232-I1 | –       | KT693572              | KT693815    | DTO 296-G6 | 140256  | KT693683              | KT693926    |
| DTO 232-I8 | –       | KT693573              | KT693816    | DTO 285-E5 | 140257  | KT693684              | KT693927    |
| DTO 232-I9 | –       | KT693574              | KT693817    | DTO 285-E6 | 140258  | KT693685              | KT693928    |
| DTO 233-A1 | –       | KT693575              | KT693818    | DTO 285-E7 | –       | KT693686              | KT693929    |
| DTO 233-A7 | –       | KT693576              | KT693819    | DTO 285-E8 | –       | KT693687              | KT693930    |
| DTO 233-A8 | –       | KT693577              | KT693820    | DTO 296-F7 | –       | KT693688              | KT693931    |
| DTO 233-C1 | –       | KT693578              | KT693821    | DTO 296-F8 | –       | KT693689              | KT693932    |
| DTO 233-C6 | –       | KT693579              | KT693822    | DTO 296-F9 | 140259  | KT693690              | KT693933    |
| DTO 233-E3 | –       | KT693580              | KT693823    | DTO 296-G1 | 140260  | KT693691              | KT693934    |
| DTO 233-F6 | –       | KT693581              | KT693824    | DTO 301-G5 | –       | KT693692              | KT693935    |
| DTO 233-F8 | –       | KT693582              | KT693825    | DTO 301-G6 | –       | KT693693              | KT693936    |
| DTO 233-G4 | –       | KT693583              | KT693826    | DTO 301-G9 | 140261  | KT693694              | KT693937    |
| DTO 233-H9 | –       | KT693584              | KT693827    | DTO 301-H1 | –       | KT693695              | KT693938    |
| DTO 233-I5 | –       | KT693585              | KT693828    | DTO 301-H2 | –       | KT693696              | KT693939    |
| DTO 234-A2 | –       | KT693586              | KT693829    | DTO 301-H3 | –       | KT693697              | KT693940    |
| DTO 234-B7 | –       | KT693587              | KT693830    | DTO 300-I2 | 140262  | KT693698              | KT693941    |
| DTO 234-C7 | –       | KT693588              | KT693831    | DTO 300-I3 | 140263  | KT693699              | KT693942    |
| DTO 234-D7 | –       | KT693589              | KT693832    | DTO 300-I4 | –       | KT693700              | KT693943    |
| DTO 234-E6 | –       | KT693590              | KT693833    | DTO 300-I5 | –       | KT693701              | KT693944    |



**Table 2** continued

| Isolate    |         | GenBank accession no. |             | Isolate    |         | GenBank accession no. |             |
|------------|---------|-----------------------|-------------|------------|---------|-----------------------|-------------|
| DTO no.    | CBS no. | ITS                   | <i>RPB2</i> | DTO no.    | CBS no. | ITS                   | <i>RPB2</i> |
| DTO 234-F4 | –       | KT693591              | KT693834    | DTO 301-F7 | 140264  | KT693702              | KT693945    |
| DTO 234-G2 | –       | KT693592              | KT693835    | DTO 301-F8 | –       | KT693703              | KT693946    |
| DTO 234-G9 | –       | KT693593              | KT693836    | DTO 301-F9 | 140265  | KT693704              | KT693947    |
| DTO 316-G9 | –       | KT693594              | KT693837    | DTO 300-I8 | –       | KT693705              | KT693948    |
| DTO 316-H1 | –       | KT693595              | KT693838    | DTO 300-I9 | –       | KT693706              | KT693949    |
| DTO 316-H2 | –       | KT693596              | KT693839    | DTO 301-A6 | –       | KT693707              | KT693950    |
| DTO 316-H3 | –       | KT693597              | KT693840    | DTO 301-A7 | –       | KT693708              | KT693951    |
| DTO 316-H4 | –       | KT693598              | KT693841    | DTO 301-A8 | –       | KT693709              | KT693952    |
| DTO 316-H5 | –       | KT693599              | KT693842    | DTO 301-F4 | 140266  | KT693710              | KT693953    |
| DTO 316-H6 | 140244  | KT693600              | KT693843    | DTO 302-E1 | 140267  | KT693711              | KT693954    |
| DTO 316-H7 | 140245  | KT693601              | KT693844    | DTO 302-E2 | –       | KT693712              | KT693955    |
| DTO 316-H8 | –       | KT693602              | KT693845    | DTO 302-E3 | –       | KT693713              | KT693956    |
| DTO 316-I1 | 140246  | KT693603              | KT693846    | DTO 302-E9 | –       | KT693714              | KT693957    |
| DTO 316-I2 | –       | KT693604              | KT693847    | DTO 302-F1 | 140268  | KT693715              | KT693958    |
| DTO 316-I3 | –       | KT693605              | KT693848    | DTO 302-F2 | 140269  | KT693716              | KT693959    |
| DTO 316-I4 | –       | KT693606              | KT693849    | DTO 302-F7 | –       | KT693717              | KT693960    |
| DTO 316-I5 | –       | KT693607              | KT693850    | DTO 302-F8 | –       | KT693718              | KT693961    |
| DTO 316-I6 | –       | KT693608              | KT693851    | DTO 302-H8 | –       | KT693719              | KT693962    |
| DTO 316-I7 | –       | KT693609              | KT693852    | DTO 302-H9 | –       | KT693720              | KT693963    |
| DTO 316-I9 | –       | KT693610              | KT693853    | DTO 302-I1 | –       | KT693721              | KT693964    |
| DTO 317-A1 | –       | KT693611              | KT693854    | DTO 302-I2 | –       | KT693722              | KT693965    |
| DTO 317-A2 | –       | KT693612              | KT693855    | DTO 302-G3 | –       | KT693723              | KT693966    |
| DTO 317-A3 | –       | KT693613              | KT693856    | DTO 302-H1 | –       | KT693724              | KT693967    |
| DTO 317-A4 | –       | KT693614              | KT693857    | DTO 302-H2 | –       | KT693725              | KT693968    |
| DTO 317-A5 | –       | KT693615              | KT693858    | DTO 302-H3 | –       | KT693726              | KT693969    |

obtained from CFU's on agar plates after culturing biomass of the substrates. They represent a small number of all the CFU's which phenotypically resembled *Aureobasidium* species. For example 7–10 isolates were studied per sample in set 5 (Table 5), while the total amount of the counted *Aureobasidium* CFU's on MEA and DG18 was up to  $9 \times 10^3$  per sampled surface (van Nieuwenhuijzen et al. publication in progress).

#### Phenotypic diversity of *Aureobasidium* strain DTO 217-G5

The macromorphology of various inoculations of DTO 217-G5 (= CBS 140241) were compared to study the limitations of a phenotypic classification method for *Aureobasidium* species. The ITS barcode of isolate DTO 217-G5 and all its inoculations were

identical. Based on this data, the isolate was identified as *Aureobasidium melanogenum* (Supplementary Data Fig. 1). The studied colonies of DTO 217-G5 (Fig. 2) were considered to be pure single strains since they were obtained as CFU's after plating a serial diluted yeast-like cell suspension. After the first transfer of four of the CFU's, which had different phenotypic characteristics on OA, to MEA P/S plates all colonies showed dark pigmentation and aerial hyphae in the margin and a more or less equal colony diameter (at 6 days of incubation). The colony texture, degree of pigmentation and mycelial production varied. The colonies after another three consecutive times of transfer and incubation showed more variation in their macromorphology. Although almost all examined cultures showed dark pigmentation, the degree varied widely and was even absent in one culture. Also the colony surface area and appearance



**Table 3** *Aureobasidium* and related fungal strains used for molecular identification

| Species name   | Strain no.                 | Source   | Locality                           | GenBank assesion no.                |             |
|--|----------------------------|--|------------------------------------|-------------------------------------|-------------|
|  |                            |  |                                    | ITS                                 | <i>RPB2</i> |
| <i>Aureobasidium leucospermi</i>                           | CBS 130593 (epiT)          | Leaves of <i>Leucospermum conocarpodendron</i>   | South Africa                       | KT693727                            | KT693970    |
| <i>Aureobasidium melanogenum</i>                           | CBS 105.22 (T)             | –  | –                                  | KT693729                            | KT693972    |
| <i>Aureobasidium melanogenum</i>                           | CBS 110374                 | Public fountain                                  | Thailand, Bangkok                  | KT693728                            | KT693971    |
| <i>Aureobasidium namibiae</i>                              | CBS 147.97 (T)             | Dolomitic marble                                 | Namibia, Namib Desert              | KT693730                            | KT693973    |
| <i>Aureobasidium proteae</i>                               | CBS 114273 (epiT)          | Leaves of <i>Protea</i> cv. ‘Sylvia’             | South Africa                       | KT693731                            | KT693974    |
| <i>Aureobasidium proteae</i>                               | CBS 111973                 | Leaves of <i>Protea</i> cv. ‘Sylvia’             | South Africa                       | KT693732                            | KT693975    |
| <i>Aureobasidium pullulans</i>                             | CBS 584.75 (NT)            | <i>Vitis vinifera</i> , fruit                    | France, Beaujolais, Beaujeu        | KT693733                            | KT693976    |
| <i>Aureobasidium pullulans</i>                             | CBS 100280                 | Salt pan   | Slovenia                           | KT693734                            | KT693977    |
| <i>Aureobasidium subglaciale</i>                           | CBS 123387 (T)             | Subglacial ice from sea water                    | Norway, Svalbard, Kongsvegen       | KT693735                            | KT693978    |
| <i>Aureobasidium subglaciale</i>                           | CBS 123388                 | Glacial ice from sea water                       | Norway, Svalbard, Kongsvegen       | KT693736                            | KT693979    |
| <i>Aureobasidium thailandense</i>                          | CBS 133856, NRRL 58539 (T) | Leaves of <i>Cerbera odollum</i>                 | Thailand, Nakhonratchasima         | GenBank no. absent; TreeBASE SN4236 |             |
| <i>Aureobasidium thailandense</i>                          | CBS 133857, NRRL 58543     | Wood surface                                     | Thailand, Prachuapkhirikhan        | GenBank no. absent; TreeBASE SN4236 |             |
| <i>Columnosphaeria fagi</i> ( <i>Discosphaerina fagi</i> ) | CBS 171.93                 | Leaf of <i>Populus</i>                           | United Kingdom                     | KT693737                            | KT693980    |
| <i>Kabatiella bupleuri</i>                                 | CBS 131304 (isoT)          | Dead flower stems, <i>Bupleurum gibraltarium</i> | Spain, Granada, Embalse de Canales | KT693738                            | KT693981    |
| <i>Kabatiella bupleuri</i>                                 | CBS 131303                 | Dead flower stems, <i>Bupleurum gibraltarium</i> | Spain, Granada, Presa de Quentar   | KT693739                            | KT693982    |
| <i>Kabatiella caulivora</i>                                | CBS 242.64                 | <i>Trifolium incarnatum</i>                      | U.S.A., Oregon                     | KT693740                            | KT693983    |
| <i>Kabatiella harpospora</i>                               | CBS 122914                 | Stems and leaves of <i>Viscum album</i>          | Spain, Madrid, Robledo de Chavela  | KT693741                            | absent      |
| <i>Kabatiella lini</i>                                     | CBS 125.21 (T)             | <i>Linum usitatissimum</i>                       | United Kingdom                     | KT693742                            | KT693984    |
| <i>Kabatiella microsticta</i>                              | CBS 114.64                 | <i>Hemerocallis</i> sp.                          | The Netherlands, Wageningen        | KT693744                            | KT693986    |
| <i>Kabatiella microsticta</i>                              | CBS 342.66                 | <i>Convallaria majalis</i> , dying leave         | Germany                            | KT693743                            | KT693985    |
| <i>Kabatiella zeae</i>                                     | CBS 767.71                 | Leaf of <i>Zea mays</i>                          | Germany, Kiel-Kitzeberg            | KT693745                            | absent      |
| <i>Selenophoma mahoniae</i>                                | CBS 388.92                 | Leaf of <i>Mahonia repens</i>                    | U.S.A., Colorado                   | KT693746                            | KT693987    |
| <i>Sydowia polyspora</i>                                   | CBS 750.71                 | <i>Pinus strobus</i> , twig                      | Canada, Quebec; Lac Normand        | KT693747                            | KT693988    |

*T* ex-type strain, *NT* ex-neotype strain, *epiT* ex-epitype strain, *isoT* ex-isotype strain

varied. Some colonies produced aerial hyphae at the margin and the degree of hyphal production varied between isolates. Furthermore, the slimy appearance

of the colonies which is described as cultural characteristic of *A. melanogenum* (Zalar et al. 2008), was also absent in some cultures.

**Table 4** All sequences from the cloning library identified as *Aureobasidium* and their corresponding GenBank accession numbers

| ITS clone | Accession no. | ITS clone | Accession no. | ITS clone | Accession no.         | ITS clone | Accession no.         |
|-----------|---------------|-----------|---------------|-----------|-----------------------|-----------|-----------------------|
| PRL.1.05  | KT693388      | PRL.2.02  | KT693421      | PRL.2.87  | KT693456              | PRL.3.80  | KT693487              |
| PRL.1.06  | KT693389      | PRL.2.03  | KT693422      | PRL.3.02  | KT693457              | PRL.3.82  | KT693488              |
| PRL.1.09  | KU671015      | PRL.2.05  | KT693423      | PRL.3.06  | KT693458              | PRL.3.83  | KT693489              |
| PRL.1.21  | KT693390      | PRL.2.07  | KT693424      | PRL.3.08  | KT693459              | PO.1.13   | KT693490              |
| PRL.1.25  | KT693391      | PRL.2.08  | KT693425      | PRL.3.09  | KT693460              | PO.1.37   | KT693491              |
| PRL.1.26  | KT693392      | PRL.2.22  | KT693426      | PRL.3.16  | KU671021 <sup>a</sup> | PO.1.45   | KT693492              |
| PRL.1.27  | KT693393      | PRL.2.23  | KT693427      | PRL.3.17  | KT693461              | PO.1.50   | KT693493              |
| PRL.1.30  | KT693394      | PRL.2.25  | KT693428      | PRL.3.19  | KU671022 <sup>a</sup> | PO.1.59   | KT693494              |
| PRL.1.31  | KT693395      | PRL.2.26  | KT693429      | PRL.3.21  | KT693462              | PO.1.68   | KT693495              |
| PRL.1.32  | KU671016      | PRL.2.27  | KT693430      | PRL.3.22  | KT693463              | PO.1.73   | KT693496              |
| PRL.1.33  | KT693396      | PRL.2.29  | KT693431      | PRL.3.23  | KT693464              | PO.1.75   | KU671024 <sup>a</sup> |
| PRL.1.34  | KT693397      | PRL.2.34  | KT693432      | PRL.3.24  | KT693465              | PO.1.81   | KT693497              |
| PRL.1.38  | KU671017      | PRL.2.35  | KT693433      | PRL.3.25  | KT693466              | PO.2.78   | KT693498              |
| PRL.1.39  | KT693398      | PRL.2.36  | KT693434      | PRL.3.28  | KT693467              | PO.3.05   | KT693499              |
| PRL.1.50  | KT693399      | PRL.2.37  | KT693435      | PRL.3.31  | KT693468              | PO.3.24   | KT693500              |
| PRL.1.51  | KT693400      | PRL.2.38  | KT693436      | PRL.3.32  | KT693469              | PO.3.68   | KT693501              |
| PRL.1.53  | KT693401      | PRL.2.39  | KT693437      | PRL.3.36  | KT693470              | PO.3.69   | KT693502              |
| PRL.1.54  | KT693402      | PRL.2.43  | KT693438      | PRL.3.37  | KT693471              | PO.3.81   | KT693503              |
| PRL.1.58  | KT693403      | PRL.2.44  | KT693439      | PRL.3.39  | KT693472              | PO.3.88   | KT693504              |
| PRL.1.61  | KT693404      | PRL.2.47  | KT693440      | PRL.3.42  | KT693473              |           |                       |
| PRL.1.62  | KT693405      | PRL.2.49  | KT693441      | PRL.3.43  | KT693474              |           |                       |
| PRL.1.67  | KT693406      | PRL.2.50  | KT693442      | PRL.3.44  | KT693475              |           |                       |
| PRL.1.69  | KT693407      | PRL.2.51  | KT693443      | PRL.3.47  | KT693476              |           |                       |
| PRL.1.70  | KT693408      | PRL.2.62  | KT693444      | PRL.3.51  | KT693477              |           |                       |
| PRL.1.71  | KT693409      | PRL.2.63  | KT693445      | PRL.3.54  | KT693478              |           |                       |
| PRL.1.74  | KT693410      | PRL.2.64  | KT693446      | PRL.3.55  | KT693479              |           |                       |
| PRL.1.75  | KT693411      | PRL.2.65  | KT693447      | PRL.3.56  | KT693480              |           |                       |
| PRL.1.76  | KT693412      | PRL.2.70  | KT693448      | PRL.3.57  | KT693481              |           |                       |
| PRL.1.77  | KT693413      | PRL.2.73  | KT693449      | PRL.3.65  | KT693482              |           |                       |
| PRL.1.78  | KT693414      | PRL.2.74  | KU671018      | PRL.3.66  | KT693483              |           |                       |
| PRL.1.79  | KT693415      | PRL.2.76  | KT693450      | PRL.3.68  | KU671019              |           |                       |
| PRL.1.81  | KT693416      | PRL.2.78  | KT693451      | PRL.3.70  | KU671023              |           |                       |
| PRL.1.82  | KT693417      | PRL.2.80  | KT693452      | PRL.3.71  | KU671020 <sup>a</sup> |           |                       |
| PRL.1.85  | KT693418      | PRL.2.82  | KT693453      | PRL.3.73  | KT693484              |           |                       |
| PRL.1.86  | KT693419      | PRL.2.83  | KT693454      | PRL.3.74  | KT693485              |           |                       |
| PRL.1.88  | KT693420      | PRL.2.86  | KT693455      | PRL.3.78  | KT693486              |           |                       |

<sup>a</sup> Sequences were trimmed to remove chimeric parts

### Identification of *Aureobasidium* isolates

The majority of all 222 sequenced *Aureobasidium* isolates could be unambiguously identified (Fig. 3). *Aureobasidium proteae* and *Columnosphaeria fagi*

resided in a clade with *A. pullulans* (Fig. 3) and are considered as synonyms of *A. pullulans*. The majority of the strains clustered together with the type of *A. melanogenum* (CBS 105.22<sup>T</sup>). Eleven strains had similar sequences as the type of *A. melanogenum*; however,

**Table 5** Overview of the (number of) *Aureobasidium* isolates per substrate (with or without biofinish) of each sample set. (– = not relevant)

| Sample set | Substrate                    | Biofinish present | Number of isolates | DTO isolate code (CBS number added when available)   |
|------------|------------------------------|-------------------|--------------------|--|
| Set 1      | Pine sapw. & raw lins. oil   | No                | 13                 | 212-D8, 212-F1, 212-G1, 212-I7, 213-A2, 213-A9, 214-C3, 214-C4, 214-C8, 214-D1, 214-D8, 214-D9, 228-C6   |
|            | Glass                        | –                 | 17                 | 212-H1, 212-H2, 214-E2, 214-E3, 214-F3, 214-F4, 214-G4, 214-G5, 214-I2, 214-I4, 214-I5, 215-B3, 215-B9, 215-C2, 215-C3, 215-D8, 215-E3   |
| Set 2      | Pine sapw. & raw lins. oil   | No                | 19                 | 217-F3, 217-F4, 217-F5, 217-G4 (CBS 140240), 217-G5 (CBS 140241), 217-H2, 218-A8, 218-B9, 218-D1, 218-F5, 218-F7, 218-G1, 218-G4, 218-G6, 218-G8, 218-H6, 218-H8, 218-I1, 228-D1 |
|            | Glass                        | –                 | 13                 | 218-I3 (CBS 140242), 218-I4 (CBS 140243), 219-A4, 219-B9, 219-D5, 219-E8, 219-E9, 219-G2, 219-H1, 219-I3, 219-B8, 219-F9, 219-B2   |
| Set 3      | Pine sapw. & raw lins. oil   | No                | 19                 | 232-D6, 232-D7, 232-E6, 232-E7, 232-H8, 232-I1, 232-I8, 232-I9, 233-A1, 233-A7, 233-A8, 233-C1, 233-C6, 233-E3, 233-F6, 233-F8, 233-G4, 233-H9, 233-I5                           |
|            | Glass                        | –                 | 8                  | 234-A2, 234-B7, 234-C7, 234-D7, 234-E6, 234-F4, 234-G2, 234-G9   |
| Set 4      | Spruce & raw lins. oil       | No                | 5                  | 316-G9, 316-H1, 316-H2, 316-H3, 316-H4   |
|            | Ilomba & raw lins. oil       | No                | 4                  | 316-H5, 316-H6 (CBS 140244), 316-H7 (CBS 140245), 316-H8   |
|            | Pine sapw. & raw lins. oil   | Yes               | 7                  | 316-I1 (CBS 140246), 316-I2, 316-I3, 316-I4, 316-I5, 316-I6, 316-I7  |
|            | Pine sapw. & olive oil       | Yes               | 8                  | 316-I9, 317-A1, 317-A2, 317-A3, 317-A4, 317-A5, 317-A6, 317-A7   |
|            | Pine sapw. & stand oil       | No                | 5                  | 317-A8, 317-A9, 317-B1, 317-B2, 317-B3   |
|            | Untreated pine sapw          | No                | 7                  | 317-B4, 317-B5, 317-B6, 317-B8, 317-B9, 317-C1, 317-C2   |
| Set 5      | Pine sapw. & stand lins. oil | No                | 7                  | 277-B5, 277-B6, 277-B7, 277-B8, 277-B9, 277-C1, 277-C2   |
|            | Ilomba & olive oil           | Yes               | 7                  | 277-C3, 277-C4, 277-C5, 277-C6, 277-C7, 277-G5, 277-G6   |
|            | Spruce & olive oil           | Yes               | 10                 | 277-F4 (CBS 140247), 277-F5 (CBS 140248), 277-F6, 277-F7, 277-F8, 277-F9, 277-G1, 277-G2, 277-G3, 277-G4   |
| Set 6      | Spruce & raw lins. oil       | No                | 3                  | 227-C6, 227-C7, 227-C8   |
|            | Ilomba & raw lins. oil       | No                | 3                  | 227-D3, 227-D4, 227-D5   |
|            | Pine sapw. & raw lins. oil   | No                | 2                  | 227-D7 (CBS 140249), 227-D8  |
|            | Pine sapw. & olive oil       | No                | 2                  | 227-E3, 227-E4   |
|            | Untreated pine sapw          | No                | 3                  | 227-E6, 227-E7, 227-E8   |
| Set 7      | Spruce & raw lins. oil       | No                | 4                  | 285-D3, 285-D4, 285-D5, 296-E8 (CBS 140250)  |
|            | Ilomba & raw lins. oil       | No                | 4                  | 285-D6, 285-D7 (CBS 140251), 285-D8, 285-D9  |
|            | Pine sapw. & raw lins. oil   | No                | 9                  | 285-E1 (CBS 140252), 285-E2 (CBS 140253), 285-E3, 285-E4 (CBS 140254), 296-F6, 296-G3, 296-G4, 296-G5 (CBS 140255), 296-G6 (CBS 140256)  |

**Table 5** continued

| Sample set | Substrate                  | Biofinish present | Number of isolates | DTO isolate code (CBS number added when available)   |
|------------|----------------------------|-------------------|--------------------|--|
| Set 8      | Pine sapw. & olive oil     | Yes               | 4                  | 285-E5 (CBS 140257), 285-E6 (CBS 140258), 285-E7, 285-E8   |
|            | Untreated pine sapw        | No                | 4                  | 296-F7, 296-F8, 296-F9 (CBS 140259), 296-G1 (CBS 140260)   |
|            | Spruce & raw lins. oil     | No                | 2                  | 301-G5, 301-G6   |
|            | Ilomba & raw lins. oil     | No                | 4                  | 301-G9 (CBS 140261), 301-H1, 301-H2, 301-H3  |
|            | Pine sapw. & raw lins. oil | No                | 7                  | 300-I2 (CBS 140262), 300-I3 (CBS 140263), 300-I4, 300-I5, 301-F7 (CBS 140264), 301-F8, 301-F9 (CBS 140265) |
|            | Pine sapw. & olive oil     | No                | 2                  | 300-I8, 300-I9   |
|            | Untreated pine sapw        | No                | 4                  | 301-A6, 301-A7, 301-A8, 301-F4 (CBS 140266)  |
| Set 9      | Spruce & raw lins. oil     | No                | 3                  | 302-E1 (CBS 140267), 302-E2, 302-E3  |
|            | Ilomba & raw lins. oil     | No                | 3                  | 302-E9, 302-F1 (CBS 140268), 302-F2 (CBS 140269)   |
|            | Pine sapw. & raw lins. oil | Yes               | 6                  | 302-F7, 302-F8, 302-H8, 302-H9, 302-I1, 302-I2   |
|            | Pine sapw. & olive oil     | No                | 1                  | 302-G3   |
|            | Untreated pine sapw        | No                | 3                  | 302-H1, 302-H2, 302-H3   |

these strains couldn't be confidentially resolved in the *A. melanogenum* clade (bootstrap values below 70 %, Fig. 3). The sequence variation could be fully attributed to the *RPB2* part of the concatenated sequences. These strains were therefore identified as *Aureobasidium* confer (cf.) *melanogenum*. Three clades with moderate bootstrap support (Fig. 3) did not contain any type or other reference strains and the isolates in these groups were tentatively named *Aureobasidium* sp. 1, sp. 2 and sp. 3. Sequences of strains named *K. microsticta*, *K. harpospora*, and *K. zae* were excluded from the *Aureobasidium* phylogenetic overview. *Kabatiella microsticta* was represented by two strains that were placed in two far apart clades in the phylogenetic tree while none of these strains were classified as type strain. The latter two *Kabatiella* species were closer related to the outgroup than to the other *Aureobasidium* species.

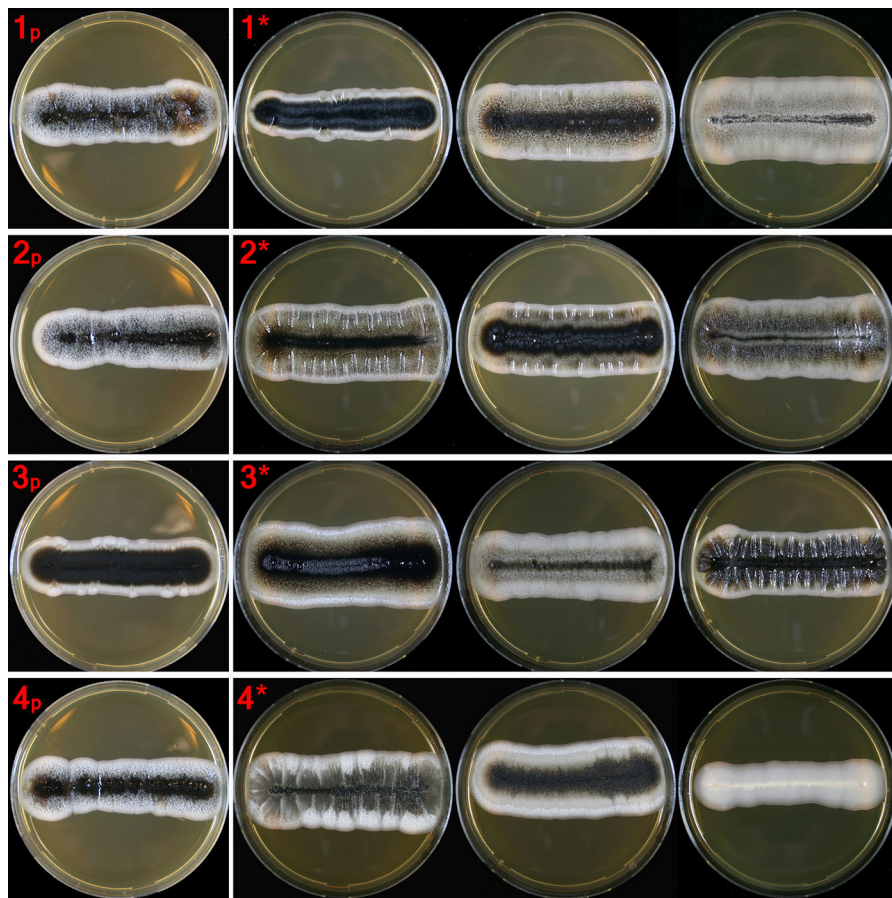
Interestingly, 18 of the 222 *Aureobasidium* isolates had ambiguous nucleotide sites in their *RPB2*

sequences. Eleven of these isolates were identified as *A. melanogenum*, one as *A. pullulans* and six as *Aureobasidium* sp. 1. The bootstrap values were above 70 % (Supplementary Data Fig. 2).

#### *Aureobasidium* species composition on stained wood surfaces

Isolates from the biofinish containing wood samples revealed that all six biofinishes contained *A. melanogenum* (Fig. 4). Other detected species were *A. leucospermi*, *A. namibiae* and *A. pullulans*. The isolates consisted of 42 *Aureobasidium* colonies that were selected after culturing biomass from biofinish containing wood. 81 % of these isolates were identified as *A. melanogenum*.

Isolates from the 27 wood specimens, which contained visual mould staining but did not have a biofinish, showed that 80 % of these wood specimens



**Fig. 2** Macromorphology of various inoculations of *A. melanogenum* strain DTO 217-G5; 1p–4p: first inoculation on MEA P/S of four single CFU's, grown at 25 °C for 6 days; 1\*–

4\*: inoculation of the same four single CFU's on MEA P/S after four consecutive transfers, grown at 25 °C for 7 days in triplicate

contained *A. melanogenum*. In addition to this species, also *A. cf. melanogenum*, *A. namibiae*, *A. pullulans*, *K. lini*, and *Aureobasidium* sp. 1, sp. 2, and sp. 3 were detected on the mould stained wood samples without biofinish (Fig. 4). These isolates consisted of 110 *Aureobasidium* colonies that were selected after culturing biomass from the sample surfaces. 60 % of these isolates were identified as *A. melanogenum*.

#### Impact of different wood and (oil-) treatments on the species composition

*A. melanogenum* was detected on 21 of the 25 wood samples of sets 4 and 6–9 after exposure at the five different sites. *A. melanogenum* was (one of) the most detected species for each substrate. Per substrate 3–6 other species were found. The species were identified as *A. leucospermi*, *A. namibiae*, *A. pullulans*, *A. cf.*

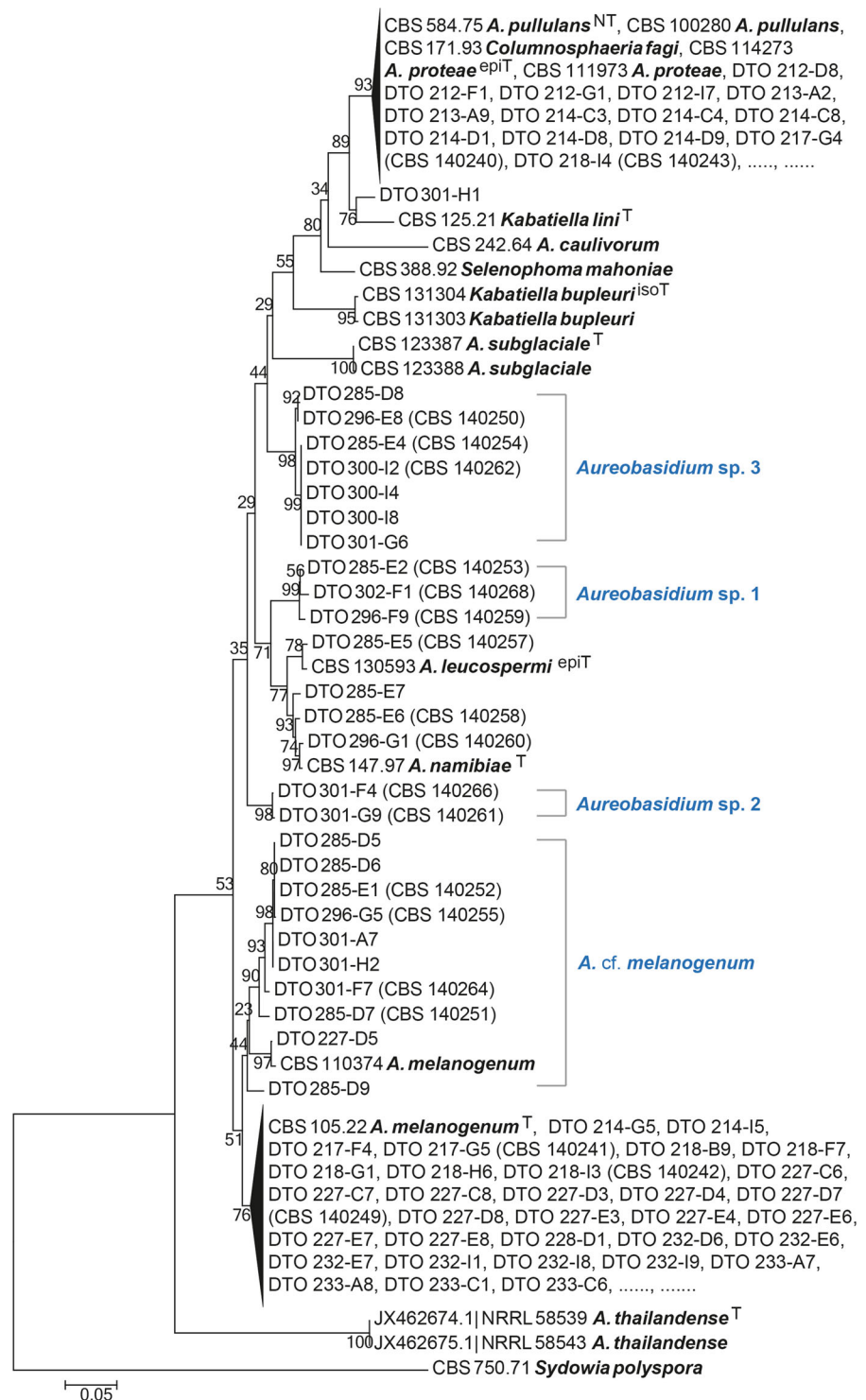
*melanogenum*, *Aureobasidium* sp. 1, *Aureobasidium* sp. 2, *Aureobasidium* sp. 3 or *K. lini*. These species were in most cases detected on one to two samples per substrate.

#### Impact of exposure sites on the species occurrence

*A. melanogenum* was detected in the sample sets exposed outdoors in the Netherlands, Cameroon, South Africa, Australia and Norway (Fig. 5). Other *Aureobasidium* species were detected as well, but were not obtained from all locations (Fig. 5). This outcome could be influenced by the limited number of isolates analysed per location. For example, 13–25 isolates were obtained from samples exposed at sites outside the Netherlands (Table 5).

Samples exposed in Australia and South Africa contained the highest *Aureobasidium* species diversity

**Fig. 3** Maximum Likelihood tree of concatenated ITS and *RPB2* sequences from outdoor *Aureobasidium* isolates and the classified reference strains. The bar indicates the number of substitutions per site. *T* ex-type strain, *NT* ex-neotype strain, *epiT* ex-epitype strain, *isoT* ex-isotype strain

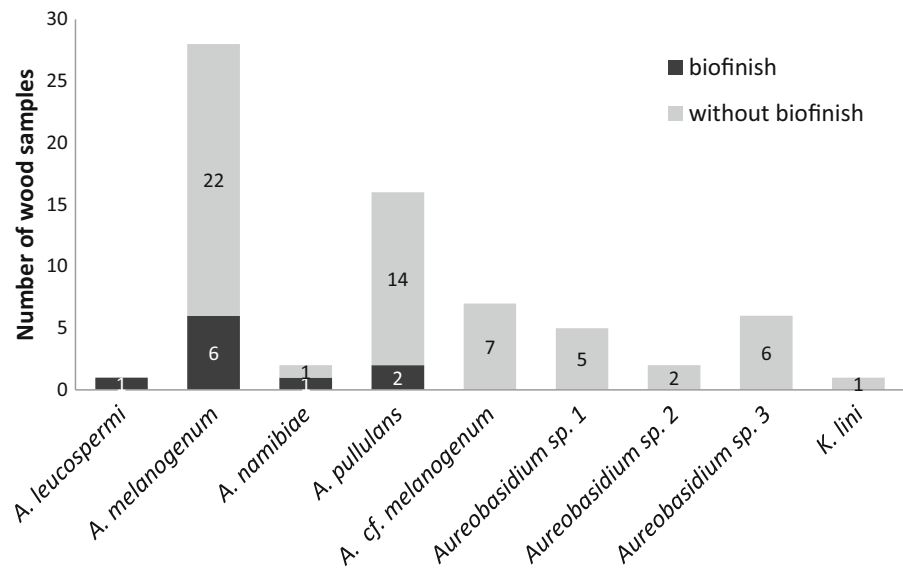


(Fig. 5). In the Netherlands, only *A. melanogenum* and *A. pullulans* isolates were detected, despite the relatively high number of substrate types (9) and identified

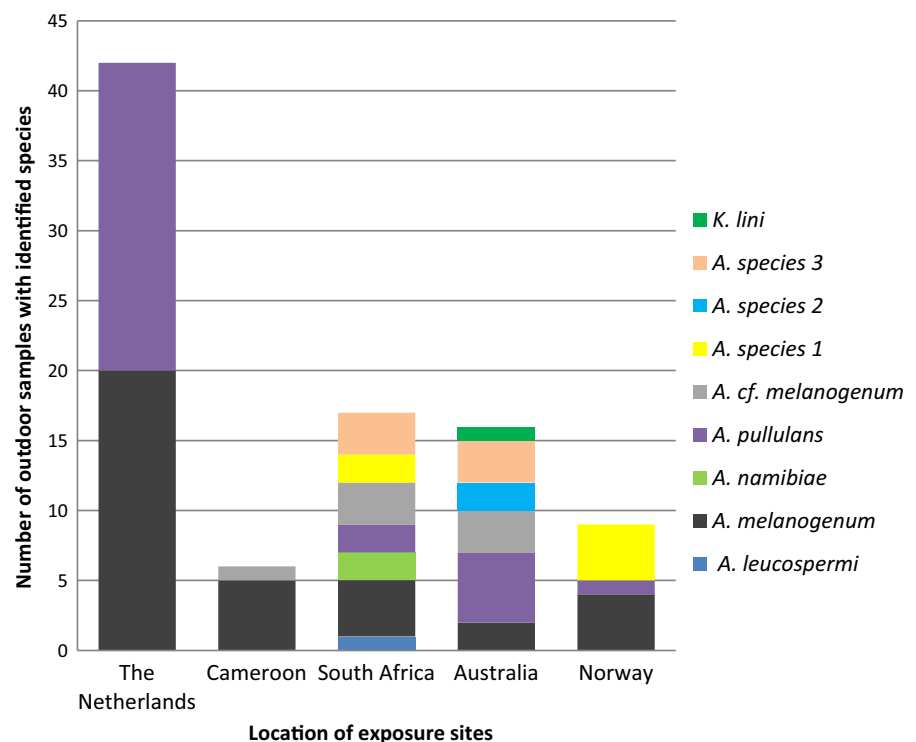
isolates (149). This indicates that the detectable species diversity of outdoor placed substrates is influenced by the location of the exposure site.



**Fig. 4** *Aureobasidium* species detected on the long-term outdoor exposed wood samples of sample sets 3–9. The total number of wood samples with a biofinish was six and without biofinish 27



**Fig. 5** Identified *Aureobasidium* species obtained from samples of sample sets 1–9 sorted by exposition site



#### *Aureobasidium* species composition on oil treated wood in time

The isolates from the pine sapwood samples treated with raw linseed oil (sample sets 1–4) showed that the

number of colonies identified as *A. pullulans* decreased over time and the number of colonies identified as *A. melanogenum* increased over time (Fig. 6). At 5 and 12 months of outdoor exposure of the samples, when mould staining on the wood surface was present, the



majority of the corresponding analysed colonies were identified as *A. melanogenum*. This in contrast to the results of the analysed colonies isolated from the reference material glass and the pine sapwood samples that had a shorter exposure time. More than 80 % of a colony set retrieved from glass was identified as *A. pullulans* regardless the exposition time.

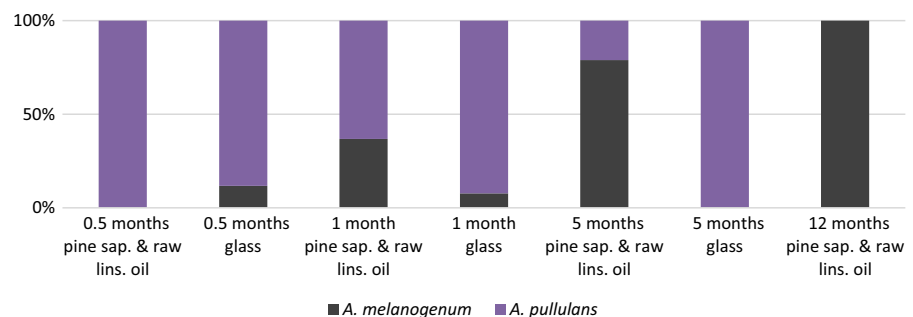
#### PCR, cloning and sequencing of biofinish DNA

In order to analyse the *Aureobasidium* species composition of biofinishes on wood without a cultivation step, cloning libraries were generated of the DNA of six biofinishes. Each of the six cloning libraries contained clones with ITS DNA that belonged to several genera. In all libraries at least one sequence was identified as *Aureobasidium* by BLASTN on the NCBI database. Most of the sequences obtained from clones with *Aureobasidium* DNA clustered together in the phylogenetic trees with either the two *A. melanogenum* or the *A. pullulans* reference strains

(bootstrap values above 63 %). A few *Aureobasidium* sequences (PRL.1.31, PRL.1.69, PRL3.16, PRL.3.19, PRL.3.70 and PO1.75) could not be identified on species level (Table 6), because they did not cluster with any of the known species. Further investigation revealed that these sequences contained parts of more than 100 nucleotides that differed largely from the reference strains.

The cloning libraries of the biofinishes on the samples treated with raw linseed oil had more than 50 % of all 62–69 clones per library identified as *Aureobasidium*. The predominant species of these cloning libraries was *A. melanogenum* (Table 6). The predominant species within the *Aureobasidium* DNA of biofinishes obtained from pine sapwood samples treated with olive oil remains unclear. Firstly the amount of clones identified as *Aureobasidium* per library was much lower, varying from 1 to 8 clones, and secondly one library showed the number of clones identified as *A. pullulans* to be equal to the ones identified as *A. melanogenum* (Table 6).

**Fig. 6** Composition of *Aureobasidium* isolates obtained from outdoor exposed oil treated pine sapwood samples or glass sheets



**Table 6** *Aureobasidium* sequences of ITS-specific clones generated from biofinishes of set 5, identified to species level

| Biofinish | Substrate                      | Number of clones identified as <i>Aureobasidium</i> |                       |                     |              | Predominant species   |
|-----------|--------------------------------|---|-----------------------|---------------------|--------------|-----------------------|
|           |                                | Total   | <i>A. melanogenum</i> | <i>A. pullulans</i> | Unidentified |                       |
| PRL.1     | Pine sapwood & raw linseed oil | 35  | 33                    |                     | 2            | <i>A. melanogenum</i> |
| PRL.2     |                                | 37  | 37                    |                     |              |                       |
| PRL.3     |                                | 38  | 35                    |                     | 3            |                       |
| PO.1      | Pine sapwood & olive oil       | 9   | 7                     | 1                   | 1            | Unclear               |
| PO.2      |                                | 1   | 1                     |                     |              |                       |
| PO.3      |                                | 6   | 3                     | 3                   |              |                       |

## Discussion

### Identification of *Aureobasidium* species

#### Morphology

Different phenotypic characteristics are described per *Aureobasidium* species (Zalar et al. 2008; Samson et al. 2010). The deviation of the general macromorphological characteristics of a strain within an *Aureobasidium* species can be explained by degeneration (Zalar et al. 2008) or phenotypic plasticity (Slepecky and Starmer 2009). Morphological changes of fungal strains on culture media after serial transfers have also been observed for other fungal species, for example *Aspergillus flavus* (Horn and Dorner 2002) and *Metarhizium anisopliae* (Ryan et al. 2002). The results in this study of the culturing of strain DTO 217-G5 showed, that phenotypic characteristics of various isolates of a single strain inoculated on the same media can differ widely. Although the original isolate DTO 217-G5 could accidentally be a mixture of strains, the studied subcultures of DTO 217-G5 were likely to be pure single strains and they still showed phenotypic diversity. Regardless the phenotypic variation of these cultures, the ITS sequences were identical. This made the use of molecular techniques, instead of morphological characteristics, essential to identify the *Aureobasidium* isolates on species level.

#### Multi-locus DNA analysis

Several isolates had ambiguous nucleotide positions in their *RPB2* sequence. The accidental presence of more than one strain in an examined isolate could explain these ambiguous nucleotide positions. In order to exclude the presence of multiple strains in a single isolate, biomass of an isolate was cultured in liquid media and after plating, separate colony forming units were used for PCR and sequencing; however, ambiguous nucleotide positions remained present in the sequences (unpublished data). Since *RPB2* is regarded as a single-copy protein coding gene (Schmitt et al. 2009; Schoch et al. 2012), more tests are needed to study this phenomenon.

The reference data set for identification of *Aureobasidium* strains contained sixteen different described taxa (Table 3) and thirteen were shown in the *Aureobasidium* phylogenetic overview (Fig. 3).

The distant relationship of *K. harpospora* (CBS 122914) to the *Aureobasidium* species and the placement of the *K. microsticta* strains (CBS 114.64 and CBS 342.66) in different phylogenetic clades is in concordance with Zalar et al. (2008) and Bills et al. (2012). The placement of *Kabatiella zeae* CBS 767.71 apart from the other *Aureobasidium* species can be supported by the outcome of a homology search of the ITS sequence of CBS 767.71 on GenBank: the best hit was *Lecythophora* sp. (KF624793.1).

### Composition of *Aureobasidium* species

The composition of *Aureobasidium* species in a biofinish on wood can be studied with different techniques. However, no single technique is available that ensures the exact result (Nevalainen et al. 2015). It is well known that techniques that are based on culturing fungi have limitations that will influence the outcome of the fungal composition (Pitkäranta et al. 2008). For example: (a) conidia might produce more colonies on an agar plate than the same amount of biomass represented by hyphae (Pitt and Hocking 2009), (b) some fungi only grow on specific culturing conditions (Pitt and Hocking 2009; Samson et al. 2010), (c) some fungi are not culturable (Pitkäranta et al. 2011; Dei-Cas et al. 2006; Blackwell 2011) and (d) some species are overgrown by other fungi in mixed samples (Samson et al. 2010). Culture independent techniques, such as targeted cloning and sequencing of DNA regions or next generation amplicon sequencing, have been used increasingly over the last years to study the composition of fungal communities, for example in the area of soil ecology (Orgiazzi et al. 2013; Clemmensen et al. 2013), wood decay (Lindner et al. 2011; van der Wal et al. 2014) and human health (Ghannoum et al. 2010; Findley et al. 2013). Although culture independent techniques are the state of the art, they also have drawbacks. Each step of a DNA based method can introduce a bias. Examples are the differences in efficiency of DNA extraction per fungal species or morphologic structure (Saad et al. 2004; Fredricks et al. 2005) and the differences in efficiency of PCR amplification per species and primer set (Vainio and Hantula 2000; Bellemain et al. 2010). In the case of amplification of the ITS region, the species dependent number of copies of the targeted rDNA region per cell (Vilgalys and Gonzalez 1990; Simon and Weiss 2008; Lindner

and Banik 2011) contributes to the different PCR amplification efficiencies. Another example of a bias is the absence of registration of taxa which are relatively scarcely present in a DNA mixture (Adams et al. 2013a; Prakitchaiwattana et al. 2004).

The origin of the fungal isolates, retrieved from the outdoor exposed specimens, is considered to be the concerned exposure site. The natural occurrence of *Aureobasidium* on outdoor exposed materials is well known and the distribution of *Aureobasidium* occurs by wind disturbance (Taylor et al. 2006), water drops (Hudson 1992; Madelin 1995) or insects (Zacchi and Vaughan-martini 2003; Pagnocca et al. 2008). Not only wood has been reported as outdoor substrate (Dix and Webster 1995; Schmidt 2006), but also other organic materials, such as leaves (Andrews et al. 2002; Woody et al. 2007), grapes (Prakitchaiwattana et al. 2004), as well as painted surfaces (Shirakawa et al. 2002; Kelley et al. 2006), plastic (Reynolds 1950; Webb et al. 2000), glass (Gorbushina and Palinska 1999; Schabereiter-Gurtner et al. 2001) and stone (Urzi et al. 2001; Ruibal et al. 2005). It should be noted that several wood samples in this study were subjected to unsterile handling and packaging.

Although analysis of bigger data sets and the use of other methods might generate a more complete view on the species compositions, the results obtained in this study indicated the predominance of *A. melanogenum* within the *Aureobasidium* population of biofinishes using a culture-based method. No deviation was observed among the different substrates of the long term exposed wood samples. The predominance of *A. melanogenum* in biofinishes on pine sapwood treated with raw linseed has been confirmed using a DNA-based method without a cultivation step. In order to confirm the indication that *A. melanogenum* is predominant within the *Aureobasidium* population of biofinishes generated on other substrates or at other exposition sites, more studies are needed. Also the potential predominance of this species within the entire fungal population of biofinishes should be investigated further. Next to *Aureobasidium* other wood stain fungi, such as species of *Cladosporium* and *Sydowia*, are to be expected (Schmidt 2006; Viitanen and Ritschkoff 2011). The use of a next generation sequencing approach, next to the culturing and PCR cloning method as used in this study, will allow a detailed compositional analysis (van Nieuwenhuijzen et al., publication in progress).

## The assets of *A. melanogenum* in biofinish formation

The finding of *A. melanogenum* as native of biofinishes on oil treated wood is a first step in understanding and controlling biofinish formation. More research is recommended on the growth mechanisms of biofinishes. The deposition (natural inoculation), attachment, survival and reproduction of fungal fragments on the oil treated wood surface are all development steps of biofinish growth outdoors.

The natural inoculation of the substrate will be influenced by the natural occurrence of certain species at a specific location. Although *Aureobasidium* has been detected in outdoor air with short-term air sampling (Larsen and Gravesen 1991; Beaumont et al. 1985; Spicer and Gangloff 2005; Pyrri and Kapsanaki-Gotsi 2007) and outdoor located sedimentation plates (Urzi et al. 2001; Adams et al. 2013b), the occurrence of some *Aureobasidium* species seem to depend on the specific outdoor location. Both *A. melanogenum* and *A. pullulans* are widely spread and might be globally present species. *A. melanogenum* isolates in this study originated from five widespread locations and this species has been isolated outdoors by others in South Africa (CBS 131917, isolated by Van der Walt), Japan, Thailand and Norway (Zalar et al. 2008). Strains of *A. pullulans* originated from the Netherlands, South Africa, Australia and Norway (this study) and at least five other countries (Zalar et al. 2008). The absence of this species in the isolates originating from Cameroon could be explained by the relative low number of isolates (Fig. 5). In contrast, the widespread occurrence of various other *Aureobasidium* species is less likely, because the relative high number of isolates from the Netherlands only consisted of *A. melanogenum* and *A. pullulans*.

The results in this study on short term exposed samples indicated that in the first weeks of outdoor exposure *A. pullulans* was more present than *A. melanogenum* on raw linseed oil treated wood as well as on glass. This did not disturb the predominance of *A. melanogenum* in a later stage of the biofinish formation. Especially since composition of *Aureobasidium* species in time seemed different on glass, the dominant influence of developments steps other than deposition seems likely.

Thus far no data has been found as to why *A. melanogenum* is predominant within the

*Aureobasidium* population of biofinishes on oil treated wood. It is currently unknown whether *A. melanogenum* is better in attachment, survival and/or reproduction on outdoor wood surfaces than other *Aureobasidium* species.

With respect to attachment: the biosynthesis of pullulan, an extracellular polymeric substance (EPS) adhesive, is described for at least four *Aureobasidium* species (Gostinčar et al. 2014) and also the production of other EPS, such as  $\beta$ -glucan and acidic polysaccharides, by different *Aureobasidium* species is known (Leal-Serrano et al. 1980; Hamada and Tsujisaka 1983; Yurlova and de Hoog 1997; Lotrakul et al. 2013).

Obviously, the production of melanin by *Aureobasidium* seems to be involved in its survival (Rättö et al. 2001; Hernández 2012; Nosanchuk and Casadevall 2003; Ruan et al. 2004; Paolo et al. 2006; Kogej et al. 2007). However, it is currently unknown whether *A. melanogenum* has an overall higher melanin content in comparison to the other species as may be suggested by its name. Genetic evidence, based on the presence of the number of genes possibly related to melanin synthesis, in an *A. melanogenum* strain (CBS 110374) and other full genome-sequenced *Aureobasidium* strains (Gostinčar et al. 2014) does not indicate obvious differences between the *Aureobasidium* species. Not only the amount, but also the type of melanin, that is produced by each species and the impact of different melanin types on specific stressors (e.g. UV, oxidizing agents) needs to be unravelled to understand the role of melanin. This requires an extensive investigation since many complicating factors are involved such as the difference in pigmentation of various isolates of a single *A. melanogenum* strain (Fig. 2), the existence of many other colours besides black in pigments resulting from melanin (Langfelder et al. 2003; Pal et al. 2014), the impact of exposure conditions on the amount of (unspecified) melanin produced by a single strain (Hernández and Evans 2015a, b) and the inability of easy melanin quantification methods, such as spectrophotometric measurements, to determine the type of melanin (Pal et al. 2014).

Next to survival, organisms need to multiply in order to support dark mould staining. Substrates are considered to play a role in this. One of the factors influenced by substrates is the availability of nutrients for fungal growth (van Nieuwenhuijzen et al. 2015).

For example, Horvath et al. (1976) presumed that nutrients for *A. pullulans* formation on wood substrates are derived from the wood. Schoeman and Dickinson (1997) also concluded that this species uses nutrients derived from wood, in particularly the products of lignocellulosic photo degradation at weathered wood surfaces. However one should keep in mind that these referred studies were performed before the recognition of *A. pullulans* and *A. melanogenum* as separate species. Next to the wood also additional materials such as oil in the case of biofinishes on oil treated wood (van Nieuwenhuijzen et al. 2013, 2015) or the attracted organic matter such as pollen (Hudson 1992) might be used for growth of *Aureobasidium*. Possibly the nutrients on oil treated wood are more favourable for *A. melanogenum* than other *Aureobasidium* species. More research is needed to understand the impact of substrates on the biofinish population.

## Conclusions

The culture based study showed the common presence of *A. melanogenum* in biofinishes that were naturally formed outdoors on oil treated wood. This fungus was also commonly found on wood samples with non-biofinish mould staining. On most of the outdoor exposed wood samples that contained stained surfaces, *A. melanogenum* was isolated, regardless the type of (oil) treatment or wood species. *A. melanogenum* was detected on samples of all five widespread exposure sites. Other *Aureobasidium* species were detected on the wood samples as well, including several potentially new species in the case of the non-biofinish samples. The results indicated that the diversity of culturable *Aureobasidium* species depends on the geographical location of the exposure site. Larger data sets for these and other locations will be required to allow more defined conclusions. ITS-specific PCR, cloning and sequencing of biofinish DNA confirmed the predominance of *A. melanogenum* within the *Aureobasidium* population of biofinishes generated in the Netherlands on pine sapwood samples treated with raw linseed oil. To allow a detailed composition analysis of the entire fungal population of biofinishes, the use of data obtained with culturing, PCR cloning and a next generation sequencing approach is suggested for future works.

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